

REVIEW

New Aspects of Accumulation and Reduction of Vanadium Ions in Ascidians, Based on Concerted Investigation from Both a Chemical and Biological Viewpoint

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ABSTRACT—Ever since high levels of vanadium was found unexpectedly in the blood cells of ascidians about 80 years ago, the mechanism of accumulation and the function of this metal in blood cells have been absorbed the interest of not only chemists but also biologists from various fields including analytical chemistry, coordination chemistry, physiology and biochemistry. Recently, this phenomenon has been the focus of concerted investigation from both a chemical and biological viewpoint. Here we summarize the most recent findings with special reference to data obtained by a combination technique involving cell fractionation for purification of a specific type of blood cell, neutron activation analysis for vanadium determination, and ESR (electron spin resonance) spectrometry for determination of chemical forms of vanadium in the blood cells.

INTRODUCTION

It was found unexpectedly by Henze about 80 years ago that ascidians contained a high level of vanadium in their blood cells [1, 2]. Many analytical chemists subsequently showed an interest, and analyzed the metal contents of species belonging to all three ascidian suborders (the Aplousobranchia, Phlebobranchia and Stolidobranchia) using newer analytical techniques [3-25]. The resulting data have shown that several species in the suborder Aplousobranchia generally have a high vanadium content, and that a significant amount of vanadium is likewise found in representatives of the Phlebobranchia, whereas species of the suborder Stolidobranchia contain relatively smaller amounts of vanadium, but a high level of iron. Webb [5] first pointed out that high concentrations of vanadium in ascidian species were correlated with certain evolutionary traits in the class Ascidiacea; he also predicted that the presence of vanadium was a primitive characteristic which had

been lost in the more specialized families, based on earlier data on the vanadium and iron contents of ascidians already examined. Endean [26, 27] reported that ascidian species belonging to the Pyuridae concentrated iron instead of vanadium. He assumed that since the ascidians are an intermediate group between the invertebrates and vertebrates, the animals were the key for resolving how transition metals were selected by organisms. Swinehart *et al.* [22] also suggested that these species might indeed represent animals that were in transition between the vanadium and iron "users". This proposal has brought particular interest from a wide area of investigation.

REEXAMINATION OF METAL CONTENTS WITH NEUTRON ACTIVATION ANALYSIS

We have also taken interest in the fact that the ascidians are the only organisms in animal kingdom to accumulate vanadium at a high level. Although animal feeding studies has suggested that the vanadium is an essential element for living organisms [28] and biochemical studies revealed

accidentally that vanadium in a V oxidation state was a potent inhibitor of Na-K-ATPase [29, 30], its physiological roles have not yet been clarified in detail. The use of ascidians, which accumulate high levels of vanadium, as study materials would be valuable for helping to resolve the mechanism of metal accumulation against a concentration gradient as high as seven orders of magnitude, as will be mentioned below. For studying this problem, we were fortunate to have access to neutron activation analysis which is the most sensitive technique for detection of vanadium (Fig. 1), and was available at the Institute for Atomic Energy of Rikkyo University. At the commencement of the study, we first intended to re-determine the vanadium content in several tissues of ascidians employing the same analytical method of the neutron activation analysis and the same pretreatments used for the determination. The data obtained from 15 species of solitary ascidian belonging to

the suborders Phlebobranchia and Stolidobranchia agreed substantially with previous reports of high vanadium levels in the family Ascidiidae, the highest amounts being present in blood cells. However, in contrast with former studies, it is important to note that significant levels of vanadium were detected in all tissues of all species, even those belonging to the suborder Stolidobranchia, and that the content of iron did not vary sharply between the two suborders studied, in contrast with vanadium content. In other words, we cannot conclude that the relative concentrations of two metals in different ascidian subfamilies reflects phylogeny [31].

Although niobium (Nb) [32, 33], titanium (Ti) [6, 13], chromium (Cr) [13, 34] and tantalum (Ta) [33] have been reported to be present in ascidian tissues besides vanadium, the reproducibility of data indicating the presence of these metals in ascidians has been poor up to the present time.

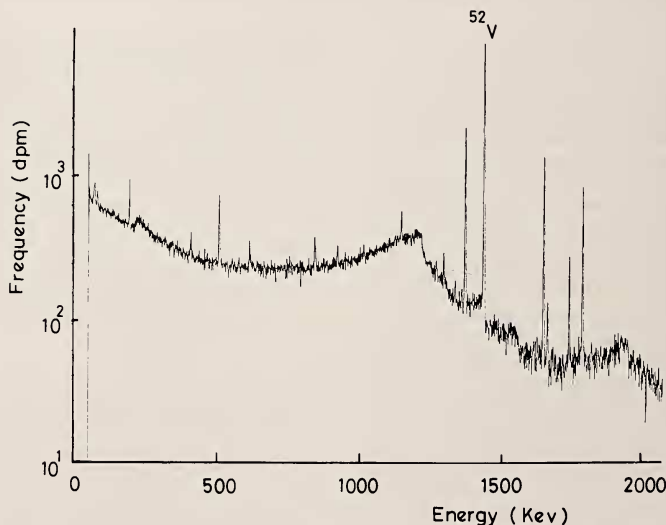


Fig. 1. γ -ray spectrometry of ascidian blood cells after irradiation with thermal neutrons at the Institute for Atomic Energy, Rikkyo University. Clear photopeak of ^{52}V , produced in the irradiated sample, can be seen.

COMBINATION TECHNIQUES OF CELL FRACTIONATION AND NEUTRON ACTIVATION ANALYSIS FOR IDENTIFICATION OF TRUE VANADOCYTES

Seawater is reported to contain dissolved vanadium ion at a concentration of 35 nM [35, 36], yet some ascidians accumulate the ion in their blood cells to a maximum concentration of 150 mM [31, 37, unpubl. data], which is more than four million times higher than that in seawater. Ascidian blood cells are classified into six to nine types based on their morphology [26, 27, 38, 39]. Among these, the morula cells (Fig. 2A), light green in color and containing several vacuoles, have been thought to be involved in the accumulation of vanadium ion and have called "vanadocytes" [5, 11, 16, 27], since the color of the cells resembles that of vanadium complex, and dense granules are observed in the cells after fixation with osmium tetroxide, which may be deposits of vanadium [16, 17, 19, 27, 40, 41]. Several investigators using X-ray microanalysis have claimed recently that the morula cells contain little or no vanadium ion. Mainly de Vincentiis's group found more vanadium ion associated with the vacuolar membranes of granular amoebocytes, signet ring cells, and compartment cells than in the vacuoles of the morula cells of *Phallusia mammillata* and *Ciona intestinalis*. Morula cells of *P. mammillata* contained very little or no vanadium ion, and granular amoebocytes of *C. intestinalis* contained more vanadium ion than

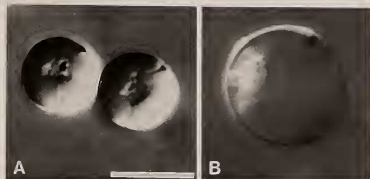


FIG. 2. Differential interference contrast photomicrographs of morula cells (A) and signet ring cell (B) in *Ascidia ahodori*. Morula cell appears typical bery-like shape. Signet ring cell is characterized by a single and fluid filled vacuole which displaced the nucleus and cytoplasm to the periphery of the cell. Scale bar indicates 10 μ m.

the morula cells [42–47]. Ultrastructural observation, however, can only provide limited data on which blood cells are the true vanadocytes because of artifacts resulting from the preparation method.

Therefore, we have attempted to identify the vanadocytes among several kinds of blood cell using a combination techniques consisting of Ficoll density gradient centrifugation and neutron activation analysis. Each kind of blood cell has a specific gravity, and consequently, a pure subpopulation of a single type of blood cell can form a separate layer depending on its specific gravity after density gradient centrifugation on Ficoll type 400, as shown in Figure 3. Each subpopulation is subjected to analysis of its vanadium content by neutron activation analysis. Following this tech-

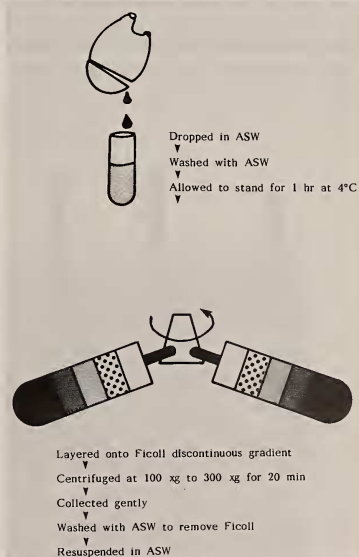


FIG. 3. Experimental scheme for density gradient centrifugation in order to obtain a pure subpopulation of each blood cell type. Ficoll type 400 was dissolved in the artificial seawater (ASW) at four different concentrations and discontinuous density gradients were prepared. Then, the cells were layered onto the gradients and were centrifuged.

nique, blood cells were partitioned into four discrete layers: Layer 1 contained a relatively small number of compartment cells and amoebocytes. Layer 2 contained mostly compartment cells (84.2%). Layer 3 contained more than 90% morula cells. Layer 4 contained 67% morula cells and 31% signet ring cells. Neutron activation analysis revealed that the vanadium level was highest in layer 4. The pattern of distribution of vanadium was compared with those of the blood cells in Figure 4. This pattern was very similar to that of the signet ring cells, but was clearly different from that of the morula cells and the other cell types. In other words, it was apparent that the vanadocytes are the signet ring cells, not the morula cells. The signet ring cell has a single, large fluid-filled vacuole containing granules, as shown in Figure 2B. The nucleus and cytoplasm are displaced by the vacuole to the periphery, giving the cell its characteristic signet ring-like appearance [48].

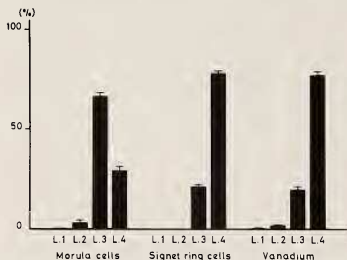


FIG. 4. Comparison of the patterns of distribution of morula cells and signet ring cells with that of vanadium ions after density gradient centrifugation. The pattern of distribution of vanadium is similar to that of signet ring cells but is different from that of morula cells.

CHEMICAL FORMS OF VANADIUM IN ASCIDIAN BLOOD CELLS

The chemical form of vanadium present in ascidian blood cells have long been a subject of discussion. Following Henze's reports [1, 2], it was believed that the vanadium was present in the form of a nitrogenous compound including sulfuric acid, well known as haemovanadin [12, 19, 49]. Recently,

it has become clear that the vanadium ion in seawater is the vanadate(V) anion [50] whereas that in ascidian blood cells is reduced to vanadyl cations, VO^{2+} and/or V^{3+} [22, 48, 51–58]. Therefore, it can be assumed that some reducing agents for reducing of vanadate ion to vanadyl form must exist in the blood cells. Several years ago Kustin's group reported the successful isolation of a reducing agent from the blood cells of *Ascidia nigra* and *Ciona intestinalis* under a reducing atmosphere [37, 59]. This substance, named tunichrome, can readily reduce vanadate ion to vanadyl ion and is also able to reduce $Fe(III)$ to $Fe(II)$ *in vitro*. Thereafter, Nakanishi and his co-workers directed their attention to this substance and isolated a tunichrome B-1 from the blood cells of *Ascidia nigra* under argon gas by means of centrifugal counter-current chromatography. They have verified that tunichrome B-1 consists of three alanine residues and that the reducing property is due to the presence of pyrogallol subunits, which are known to reduce vanadate(V) to vanadyl(IV) and to form complexes [60, 61]. Furthermore, this substance has been reported to emit a specific autonomous fluorescence at 532 nm and 581 nm upon excitation with blue-violet light at 488 nm, and consequently, it has been suggested that the intensity of fluorescence is indicative of the concentration of vanadium ion in blood cells because both tunichrome and vanadium have been presumed to be present at approximately equimolar concentrations based on the stoichiometry of the reaction of vanadium with tunichrome purified from *A. nigra* *in vitro*. Spectrofluorometric analysis revealed that the concentration of tunichrome was highest in morula cells, followed by compartment cells and signet ring cells in that order. Therefore, it was believed that the vanadium concentrations occurred in the same order, i.e., the morula cell was the so-called vanadocyte [62, 63].

If tunichrome is involved in the accumulation and reduction of vanadium ion in ascidian blood cells, it is necessary to confirm that vanadocytes do in fact contain both tunichrome and vanadium. As mentioned above, we have already proved that morula cells contain no vanadium, whereas signet ring cells contain a very high level of vanadium ion in the case of *A. ahodori* [48]. In order to verify

the participation of tunichrome in the accumulation of vanadium ion from seawater and its reduction in ascidian blood cells, we examined whether the signet ring cells, newly identified as vanadocytes, emit autonomous fluorescence due to the tunichrome. In recent experiments, we found that the morula cells, compartment cells and the orange pigment cells emitted autonomous fluorescence after excitation with blue-violet light, composed of line spectra at 405 nm and 435 nm and a wide spectrum at 490 nm. The fluorescence emitted from the morula cell was absorbed by an O515 barrier filter, indicating that its wavelength was longer than 515 nm. The compartment cell and orange pigment cell emitted a fluorescence longer than 475 nm and 530 nm, respectively. However, no fluorescence due to the tunichrome was detected from the signet ring cell as shown in Figure 5 [64]. The same results were obtained in a different ascidian species, *A. sydneyensis samea* [65]. At the present time, therefore, it is doubtful whether tunichrome participates in the accumulation and reduction of vanadium ion in ascidian blood cells.

VANADIUM-BINDING SUBSTANCE (VANADOBIN) EXTRACTED FROM ASCIDIAN BLOOD CELLS

On the other hand, as we have considered that the vanadium must be present as some type of complex in ascidian blood cells, we attempted to extract and purify a vanadium-binding substance from the blood cells of *Ascidia sydneyensis samea* by a combination of techniques including chromatography, neutron activation analysis and electron spin resonance spectrometry (ESR). Through this approach, we succeeded in obtaining the vanadium-binding substance by chromatographies on Sephadex G-25 and SE-cellulose under the low pH conditions [57]. This substance, named vanadobin, is colorless and can maintain the vanadium ion in the vanadyl form (VO(IV)) even under aerobic conditions. Moreover, it has an affinity for exogenous vanadate ion(V) and contains a reducing sugar. The most recent experiments have revealed that vanadobin is detectable in the signet ring cells [unpubl. data].

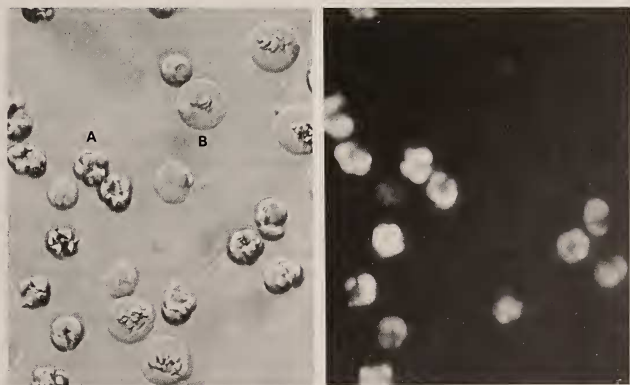


Fig. 5. Blood cells of *Ascidia ahodori* observed with a light (left) and a fluorescence microscope (right). Morula cell (A) emitted fluorescence due to tunichrome upon excitation with blue-violet light but signet ring cell (B) did not fluoresce.

NEW PHYSICAL TECHNIQUES FOR RESOLVING THE CHEMICAL FORM OF VANADIUM IN BLOOD CELLS

New physical technology, including EXAFS (extended X-ray absorption fine structure), ESR and SQUID (superconducting quantum interference device), may provide new information on chemical structure of vanadium in ascidian blood cells. These techniques have an advantage in that they can determine the intracellular oxidation state of vanadium non-invasively. Although many investigators have detected the IV oxidation state of vanadium in the blood cells using ESR spectrometry (Fig. 6) [22, 48, 54–58], whether the IV oxidation state is predominant remains unsolved. Hawkins *et al.* [55] mentioned that the Aplousobranchia had vanadium ion in the oxovanadium(IV) form, whereas the Phlebobranchia contained vanadium ion in the trivalent(III) state. Dingley *et al.* [53] reported that about 4% of the total vanadium in the cells existed in the vanadyl form. Recently, Hodgson's group, using EXAFS, has reported that the vanadium exists in the III oxidation state and binds only with water molecules in blood cells [51, 66]. Measurements with SQUID have also revealed that the predominant species of vanadium is that in the III oxidation state in blood cells of *Ascidia nigra* [67]. Views are still divided regarding the chemical forms and oxidation states of vanadium in ascidian blood cells.

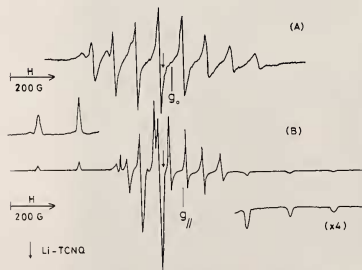


Fig. 6. ESR spectra characteristic of oxovanadium (IV) observed in the blood cells of *Ascidia ahodori*, which were measured at room temperature (A) and at 77K (B).

Besides the blood cells, vanadyl ions have been detected in the branchial basket of *Ascidia ahodori* by ESR spectrometry. Furthermore, it was observed that exogenous vanadate ions were reduced to the vanadyl form upon incubation with fragments of branchial basket, suggesting that the branchial basket may be the first organ to reduce vanadium ion, and that the reduced ion is then transferred to the blood cells [68].

PROBLEM OF LOW pH IN VANADOCYTES

The mechanism of accumulation of high levels of vanadium in ascidian blood cells has been considered to be associated with a very low pH value within the cells [5, 11, 13, 23, 26, 40, 49, 54, 69, 70]. The intracellular pH of vanadocytes has, however, been disputed. Following Henze's discovery of 1N acidity in ascidian blood cells [1, 2], 1.83 N sulfuric acid and 0.39 N acid were measured by titration method of the acid liberated upon cytolysis of the blood cells of *Phallusia mammillata* [5] and *Pyura stolonifera* [26], respectively. Dingley *et al.* [71] and Agudelo *et al.* [72] recently claimed that the methods used previously gave spurious results when applied to ascidian blood cells. They mentioned that the intracellular pH was neutral on the basis of measurements made by a new technique with improved trans-membrane equilibrium of ^{14}C -labeled methylamine. Hawkins *et al.* [73, 74] also found a neutral pH value using a non-invasive probe based on the chemical shift of ^{31}P -NMR (nuclear magnetic resonance). Conversely, Frank *et al.* [56] reported a blood cell of pH value of 1.8 based on a new finding that the ESR line width reflected accurately the intracellular pH, a method which was also non-invasive. These previous studies focused on the greenish-hued morula cells, considered to be vanadocytes, but these, of course, were not separated from the other blood cells. We believe that one of the reasons for the variation in pH values reported for ascidian blood cells is probably the measurement of pH without cell fractionation. In other words, one blood cell type among several must have a highly acidic solution within its vacuole, where the vanadium ions would be present in a reduced state. The combined technique involving cell fractionation by density

gradient centrifugation, a microelectrode for pH measurement and neutron activation analysis for vanadium measurement revealed that the signet ring cells had a low pH of 2.6 under both aerobic and anaerobic conditions and contained a high amount of vanadium in *Ascidia ahodori* [unpubl. data]. Using X-ray microanalysis with electron microscopy, Bell *et al.* [54] and de Vincentiis's group [42-46] reported the coexistence of sulfur with vanadium in blood cells. It is within the bounds of possibility that the accumulation and reduction of vanadium ion is carried out in the presence of sulfuric acid in the blood cells. Whether the vanadocyte, or more precisely speaking, the vacuole fluid of the vanadocyte, has a low pH is still a matter to be resolved in order to account for the mechanism of accumulation and reduction of vanadium ions in ascidian blood cells.

FUNCTION OF VANADIUM CONTAINED IN BLOOD CELLS

The function of such a high concentration of vanadium in ascidian blood cells remains unexplained. Endean [26, 27, 75, 76] and Smith [77, 78] proposed that the cellulose of the tunic might be produced by vanadocytes. Carlisle [21] has suggested that vanadium-containing vanadocytes can reversibly trap oxygen under conditions of low oxygen tension. Swinehart *et al.* [22] suggested the acid-producing function of vanadium, and Rowley [79] has demonstrated that the vanadium contained in vacuole works as an antimicrobial agent. However, almost all of the proposals put forward have recently become doubtful on the basis of newer experimental evidence. We maintain that any proposals concerning the function of vanadium should be reconsidered in the light of current evidence.

It is well known that transition metals including vanadium have several oxidation states and easily form coordination complexes [80-85]. Therefore, these metals have been successively revealed to be involved in enzymatic reactions and reduction-oxidation reactions in living organisms [86-89]. For example, vanadium functions as an active center in bromoperoxidase, extracted from marine algae [90, 91], and in nitrogenase purified from

bacteria [92-94]. Vanadium complex, named amavadin, was isolated from fly agaric [95]. It becomes clear that vanadium exerts an insulin-like effect on adipocytes and diabetic rats [96, 97] and inhibitory effect on carcinogenesis [98]. Moreover, the oxidation states and ionic forms of vanadium are known to be readily changeable by ascorbic acid, glutathione, cysteine and NADH (reduced nicotinamide adenine dinucleotide) [99-103], substances which are common to all living organisms.

CONCLUSION

The fact that some ascidians accumulate vanadium ion to a level exceeding four million times that present in seawater is a remarkable phenomenon. Resolution of the above issues would be a shortcut to clarifying the mechanism of accumulation of trace elements across bio-membranes and the function of transition metals in the other organisms as well as ascidians. Interdisciplinary investigation, based on marine biology, physiology, biochemistry, bioinorganic chemistry and the chemistry of natural products, are absolutely necessary for achieving these aims.

ACKNOWLEDGMENTS

I would like to express my hearty thanks to my associates for their friendly assistance and to Dr. Noriyuki Satoh, Kyoto University, for many stimulating discussion. Particular thanks are due to the staff of marine biological stations of the Asamushi, Tohoku University, the Ushimado, Okayama University, and the Misaki, University of Tokyo. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan and was also supported financially by the Japan Securities Scholarship Foundation and the Ito Science Foundation. A part of this paper was announced at the Japan-U.S. Cooperative Seminar on the theme, "Developmental Biology of the Ascidian", which was held on Aug. 22-27, 1988 at Asamushi, Aomori, Japan.

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